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Studies on Chlorogenic Acid Biosynthesis in Sweet Potato Root Tissue in Special Reference to the Isolation of a Chlorogenic Acid Intermediate[1]

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ABSTRACT

Marked polyphenol production takes place in root tissue of sweet potato, *Ipomoea batatas* Lam. cv. Norin 1, in response to slicing. A possible intermediate, tentatively termed compound V, of chlorogenic acid biosynthesis was isolated from the root tissue administrated with *t*-cinnamic acid-2-^{14}C. Compound V was proved to be an ester whose acid moiety was *t*-cinnamic acid, and the hydroxyl group-bearing moiety appeared to be a carbohydrate. Compound V was suggested to be the first intermediate after *t*-cinnamic acid involved in the chlorogenic acid biosynthetic pathway by the following three results. (*a*) label of *t*-cinnamic acid-2-^{14}C was distributed in compound V first, then transferred to chlorogenic acid and isochlorogenic acid, isomers of dicaffeoylquinic acid; (*b*) specific radioactivity of compound V increased prior to that of the fraction containing chlorogenic acid and isochlorogenic acids and decreased prior to that of the latter; and (*c*) label of compound V was efficiently incorporated into chlorogenic acid and isochlorogenic acid.

Chlorogenic acid is widely distributed among higher plants (1, 2); besides, it plays important roles in some physiological functions of plants. For example, chlorogenic acid participates in the defense action of plants (10, 11, 16, 17) and inhibits IAA oxidase (6).

Radioisotopic experiments have demonstrated that the aromatic moiety of chlorogenic acid comes from *t*-cinnamic acid. The pathway beyond *t*-cinnamic acid, however, still remains obscure, although some theories have been proposed. Levy and Zucker (12) hypothesized that 5-*O*-cinnamoylquinic acid and 5-*O*-*p*-coumaroylquinic acid were intermediates in the biosynthesis of chlorogenic acid. According to El-Basyouni and Neish (4), actual intermediates of the phenylpropanoids in lignin biosynthesis were protein-binding esters of cinnamic acid derivatives.

When sweet potato root tissue is sliced, some enzymes involved in the biosynthesis of phenolic compounds are formed (13, 14), and subsequently polyphenolic compounds, mainly composed of chlorogenic and isochlorogenic acids, isomers of dicaffeoylquinic acid, are markedly produced. By using this system, we have suggested the operation of the following two pathways in sweet potato root tissue: phenylalanine → *t*-cinnamic acid → *t*-cinnamoyl derivative → *p*-coumaroyl derivative → chlorogenic acid, and phenylalanine → *t*-cinnamic acid → *p*-coumaric acid → *p*-coumaroyl derivative → chlorogenic acid (9).

In earlier studies on chlorogenic acid biosynthesis (8, 12), we have assumed that it is difficult to isolate the chlorogenic acid intermediates due to rapid turnover and a small pool size. Therefore, two attempts were made to increase the intermediates. First, since O_2 is required to produce the hydroxyl groups of the caffeic acid moiety of chlorogenic acid, a deficiency of O_2 should result in accumulation of the intermediates in the tissue. The second is based on the hypothesis that some of the enzymes involved in chlorogenic acid biosynthesis have not yet been induced in the short incubated tissue, so that intermediates will accumulate.

In this paper, we report the finding of a possible precursor in chlorogenic acid biosynthesis in sweet potato root tissue and some of its properties.

MATERIALS AND METHODS

Sweet potatoes (*Ipomoea batatas* Lam. cv. Norin 1) were harvested in October and stored at 10 C until used. The roots were washed, the outer cortex tissues were removed, and discs, 2 mm thick and 19 mm in diameter, were prepared from the inner parenchymatous tissue, thoroughly washed with water, and blotted. One disc weighed about 0.55 g.

Reagents. *t*-Cinnamic acid-2-^{14}C was synthesized and purified by the Perkin reaction modified by Doebner (16). Melting point data and paper radioautography showed no presence of contaminants in the synthesized compound. Synthetic 3-*O*-cinnamoylquinic acid was presented by Dr. M. Zucker.

Incubation Conditions. Twenty-four discs were incubated with 12.5 μmoles of *t*-cinnamic acid-2-^{14}C (70.5 mμc per μmole) in 5 mM acetate buffer, pH 5.5, in two 9-cm Petri dishes, each of which contained 0.5 ml of acetate buffer (6.25 μmoles of the labeled compound), unless otherwise stated, at 30 C for a specified period.

Ethanol Extract Preparation. After incubation, the discs were washed with 100 ml of water, boiled in 30 ml of ethanol for 4 min, and homogenized in a blender. After addition of 50 ml of ethanol, the homogenate was boiled for 30 min and filtered with suction. The residue was washed three times with ethanol. The combined filtrate and washings were adjusted to a certain volume.

Silica Gel Column Chromatography. An aliquot of the etha-

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nol extract was evaporated with Avicel SF powder to dryness. The powder was subjected to silica gel column chromatography by the method of Hanson and Zucker (17), with slight modification in the elution solvent system. Sample was eluted from the column, using a rectilinear gradient elution system: starting solvent, 400 ml of cyclohexane-chloroform (3:7, v/v) equilibrated with 0.5 N sulfuric acid; limiting solvent, 400 ml of *t*-butyl-alcohol-chloroform (3:7, v/v) equilibrated with 0.25 N sulfuric acid. Each fraction was 5 ml.

Radioactivity Determination. An aliquot of the liquid sample not exceeding 1 ml was mixed with 10 ml of the Bray's scintillation fluid. Strips of paper after chromatography were cut into 1 cm pieces, each of which was placed in a vial containing 10 ml of the Bray's scintillation fluid. The residue, after ethanol extraction, was dried and ground in a mortar. The resultant powder was suspended in a vial with 0.4 g of the thixotropic gel powder, CAB-O-SIL, in 10 ml of the Bray's scintillation fluid. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, Model 314 E or Model 2003.

RESULTS

Time Course of Distribution of Radioactivity of *t*-Cinnamic Acid-2-^{14}C. Time course changes in distribution of radioactivity of *t*-cinnamic acid-2-^{14}C during incubation were studied using paper and silica gel column chromatographies. Radioactivity of *t*-cinnamic acid-2-^{14}C was distributed at first in compound V (tentatively called) 2 hr after administration, then appeared in chlorogenic acid, and finally in isochlorogenic acid (Fig. 1). Compound V had a Rcg value of 0.8 on silica gel column chromatography (7). Compound V eluted from the silica gel column was subjected to thin layer and paper chromatographies. These data indicated that compound V was a single compound. These results suggest that compound V is the first intermediate after *t*-cinnamic acid in the chlorogenic acid biosynthetic pathway.

Time Course of Specific Radioactivity of Metabolites of *t*-Cinnamic Acid-2-^{14}C. The ethanol extracts from discs incubated for various periods were subjected to silica gel column chromatography and, the specific radioactivities of some of the eluted compounds were determined. As shown in Figure 2, specific radioactivity of compound V increased prior to that of a fraction containing chlorogenic and isochlorogenic acids and decreased prior to that of the latter. The results strongly suggest a precursor-product relationship between compound V and chlorogenic and isochlorogenic acids.

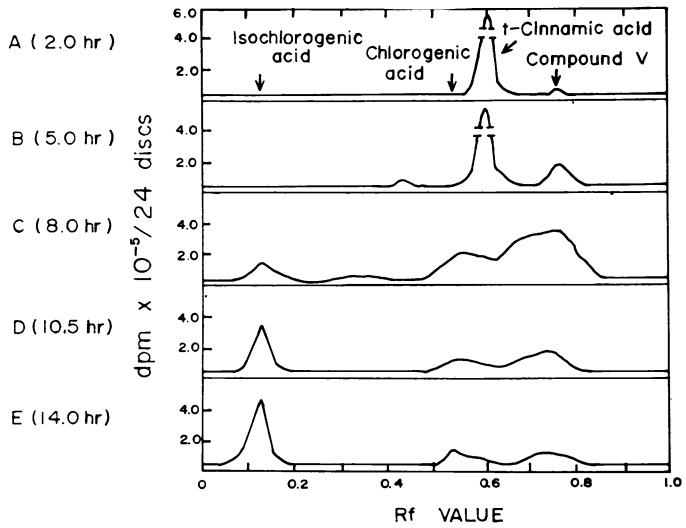

FIG. 1. Paper radiochromatograms of ethanol extracts of sweet potato root discs incubated for various periods after administration of *t*-cinnamic acid-2-^{14}C. The ethanol extracts were chromatographed on paper using 5% acetic acid as developing solvent. Arrows on chromatograms indicate the migrating positions of the individual compounds in the solvent system. A, B, C, D, and E: radiochromatograms after 2.0 hr-, 5.0 hr-, 8.0 hr-, 10.5 hr-, and 14.0 hr-incubations, respectively.

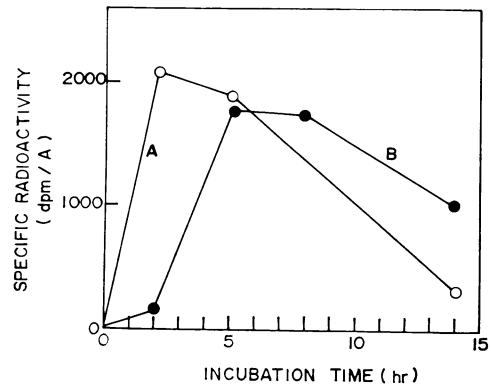

FIG. 2. Time course of specific radioactivity of metabolites of *t*-cinnamic acid-2-^{14}C. The ethanol extracts from discs incubated for various periods were subjected to silica gel column chromatography. Fractions were assayed for the contents and radioactivities. The contents were measured spectrophotometrically at maximum absorption. The specific radioactivity was expressed as dpm of 1 ml of each sample per absorbance at the maximum (278 or 328 nm of compound V or chlorogenic and isochlorogenic acids in ethanol). A: Compound V. (—○—) B: chlorogenic and isochlorogenic acids. (—●—).

Administration of Radioactive Compound V to Preincubated Discs. Radioactive compound V was prepared from 50 discs which were incubated anaerobically, by submerging them in water for a short period (8 hr), to accumulate the intermediates after administration of *t*-cinnamic acid-2-^{14}C. After incubation, an ethanol extract was prepared, from which compound V was isolated by repeating silica gel column chromatography. After examination of its purity by paper chromatography (5% acetic acid), radioactive compound V was administrated to the discs preincubated at 30 C for 12 hr. After 18 hr of incubation, the distribution of radioactivity was analyzed. The radioactivity of compound V was efficiently incorporated into chlorogenic and isochlorogenic acids (Table I). This is also supportive evidence that compound V is an intermediate in chlorogenic acid biosynthesis.

Properties of Compound V. Compound V showed maximum absorption at 278 nm in either ethanol or water and no alkaline shift, suggesting the absence of phenolic hydroxyl group(s) in its structure. Neither a phosphate nor an amino group was detected in compound V, when the Hanes-Isherwood reagent or ninhydrin reagent was sprayed on paper chromatogram. Compound V was different from synthetic 5-*O*-cinnamoylquinic acid in chromatographic behavior, and infrared spectrum. Compound V and 5-*O*-cinnamoylquinic acid showed the R_F values of 0.26 and 0.34 on silica gel thin layer chromatography (toluene-ethylformate-formic acid, 5:4:1, (v/v)), respectively. Compound V was easily hydrolyzed in 1 N alkaline solution into two components. After acidification with 5 N HCl, an aliquot of hydrolyzate was subjected to silica gel thin layer (toluene-ethylformate-formic acid, 5:4:1, (v/v)) and paper (5% [v/v] acetic acid) chromatographies. On both chromatograms, one component of compound V had the same R_F values (0.75 and 0.58 on thin layer and paper chromatograms, respectively),

Table I. *Distribution of Radioactivity of Various Fractions in Sweet Potato Root Tissue Discs after Administration of Radioactive Compound V*

Radioactive compound V was prepared from the discs which received administration of *t*-cinnamic acid-2-^{14}C. Twenty eight discs preincubated at 30 C for 12 hr were administrated with radioactive compound V (3.57×10^5 dpm, 430 as absorbance at 278 nm in 1 ml) in 1 ml of 5 mM acetate buffer, pH 5.5, and incubated at 30 C for 18 hr.

	Radioactivity	Radioactivity
	dpm × 10^5	%
Administrated	3.57	100
Unabsorbed	0.96	3
Insoluble fraction	1.36	38
CO_2	1.08	30
Ethanol extract[1]	1.03	29
Chlorogenic acid	0.21	6
Isochlorogenic acid	0.57	16

[1] Ethanol extract was subjected to paper chromatography (5% acetic acid) to obtain the fractions of chlorogenic and isochlorogenic acids.

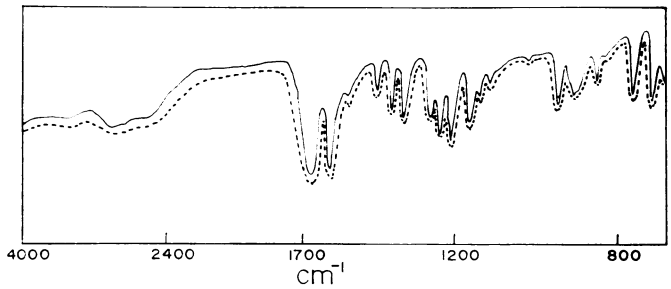

FIG. 3. Infrared spectra of aromatic component of compound V (——) and authentic *t*-cinnamic acid (– – –). Compound V was kept in 1 N NaOH at room temperature for 12 hr. Hydrolyzate was acidified with 5 N HCl and extracted with ether three times. The ether extract was evaporated to dryness and dried over P_2O_5. Spectra were recorded as KBr discs with a JASCO IR-G spectrometer.

as those of authentic *t*-cinnamic acid, and differed significantly in movement from compound V. The rest of the hydrolyzate was extracted with ether. The ether extract was evaporated to dryness. The dried ether-soluble component was analyzed by UV, infrared, and mass spectrometry (Fig. 3). All spectral data of ether-soluble component from compound V were identical with those of the authentic *t*-cinnamic acid. Thus, one component of compound V was proved to be *t*-cinnamic acid.

On the other hand, another component of compound V was presumed to be carbohydrate, based on chromatographical behavior of compound V (lower R_F value and easily carbonized by sulfuric acid on silica gel thin layer chromatogram), infrared spectrum (strong absorptions of hydroxy group and carbonyl group at 3400 cm^{-1} and 1700 cm^{-1}, respectively) and nuclear magnetic resonance spectrum (a large broad signal extinguished with D_2O around τ 5.8).

Distribution of Label of Compound V. Compound V was isolated from discs administrated with *t*-cinnamic acid-2-^{14}C and hydrolyzed in 1 N alkaline solution to yield the above two components (*t*-cinnamic acid and another component). The label of compound V was strictly confined to *t*-cinnamic acid. This also strengthens the view for occurrence of compound V as the first chlorogenic acid intermediate after *t*-cinnamic acid.

DISCUSSION

Careful attention was made to avoid contamination of microorganisms and nonenzymatic changes of administrated radioactive *t*-cinnamic acid. Thus, compound V isolated from our preparation has been proved to be neither a metabolite of contaminating microorganisms nor an artificial product of *t*-cinnamic acid-2-^{14}C.

Levy and Zucker (12) obtained evidence for the occurrence of a trace amount of a conjugate of *t*-cinnamic and quinic acids in potato tuber slices that had been maintained in the dark in a solution containing the above two components. Finally, they proposed that chlorogenic acid was synthesized through *5-O*-cinnamoylquinic acid and *5-O-p*-coumaroylquinic acid (12). Hanson (8), however, failed in finding *5-O*-cinnamoylquinic acid in potato tuber slices and in demonstrating its involvement in chlorogenic acid biosynthesis. Using sweet potato root discs, we investigated repeatedly whether or not cinnamoylquinic acid was detected with silica gel column chromatography developed by Hanson and Zucker (7). No cinnamoylquinic acid, however, was detected in the eluted fractions.

On the other hand, we isolated a new possible intermediate, compound V, of chlorogenic acid biosynthesis in sweet potato root tissue. Compound V is thought to be a precursor of chlorogenic acid, from the following experimental results: label of *t*-cinnamic acid appeared in compound V first and was possibly transferred to chlorogenic and isochlorogenic acids during incubation, the specific radioactivity of compound V increased, then decreased prior to that of a fraction containing chlorogenic and isochlorogenic acids; radioactive compound V was efficiently incorporated into chlorogenic and isochlorogenic acids. The data are consistent with hypothesis that compound V is the first intermediate after *t*-cinnamic acid in the chlorogenic acid biosynthetic pathway, but the possibility that compound V is located on a by-pass has not been ruled out completely.

Compound V was easily hydrolyzed into two components in alkaline solution. One component was identified as *t*-cinnamic acid. The nonaromatic moiety has not yet been identified, but it is neither quinic nor shikimic acid. According to the data available at present, it is probably a carbohydrate. Thus, it has been shown that compound V is a conjugate of *t*-cinnamic acid and some sugar different from quinic or shikimic acid. On the other hand, the structure of chlorogenic acid has been proved to be *5-O*-caffeoylquinic acid. Therefore, the nonaromatic moiety of compound V would have to be replaced with quinic acid in a later step of chlorogenic acid biosynthesis.

Time course analysis indicated that chlorogenic acid was not the final product but was metabolized to isochlorogenic acid in sweet potato root tissue. This result is consistent with the fact that isochlorogenic acid is present in a larger amount than chlorogenic acid in incubated sweet potato root discs.

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